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## Autophagy in Alcoholic Liver Disease, Self-eating Triggered by Drinking

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### Abstract

Macroautophagy (autophagy) is an evolutionarily conserved mechanism. It is important for normal cellular function and also plays critical roles in the etiology and pathogenesis of a number of human diseases. In alcohol-induced liver disease, autophagy is a protective mechanism against the liver injury caused by alcohol. Autophagy is activated in acute ethanol treatment but could be suppressed in chronic and/or high dose treatment of alcohol. The selective removal of lipid droplets and/or damaged mitochondria is likely the major mode of autophagy in reducing liver injury. Understanding the dynamics of the autophagy process and the approach to modulate autophagy could help finding new ways to battle against alcohol-induced liver injury.

### Keywords

Alcoholic liver disease; Ethanol; Autophagy; Mitophagy; Lipophagy; Mitochondria

### Essentials of Autophagy

Autophagy is a cellular degradation process [1, 2]. There are three different autophagic processes: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA), which differ in their physiological function and the way by which cytoplasmic materials are delivered to the lysosome. In macroautophagy cellular materials are carried by the double-membraned autophagosomes to the lysosome. Macroautophagy, referred to as autophagy hereafter, may be responsible for the majority of autophagy activity in normal cells and can degrade large cellular components other than proteins, such as mitochondria, lipid droplets or the endoplasmic reticulum (ER) membranes [1]. In mammals, autophagy is well studied in the liver [3]. In fact, most pioneering works in this field were carried out during the characterization of hepatic autophagy.

The origin of the autophagosomal membrane is not quite clear. Various models have been proposed [4]. These include the model that the membrane is synthesized *de novo* and the

model that the membrane is derived from pre-existing cellular membranes such as the (ER) [5, 6], the Golgi complex [7], the mitochondria [8] and the plasma membrane [9]. Recent studies suggest that ER is the most plausible candidate for the initial membrane source and/or the platform for autophagosome formation following amino acid starvation [10]. It has to be noted that other membranes could also contribute to autophagosome formation and/or maturation at the early or later phase of the process. This may be particularly meaningful in selective autophagy in which specific subcellular organelles are targeted.

The molecular mechanism involved in the activation of autophagy and the formation of autophagosome is largely derived from the discovery of autophagy related genes (ATGs) in the yeast, most of which have corresponding mammalian homologues [2]. Autophagy is regulated by complicated signal transduction pathways. Among them, mTORC1 and AMPK are sensors of intracellular signaling, which cause the suppression or stimulation of autophagy, respectively. The core autophagy machinery is composed of Atg molecules, which form several complexes important for autophagy induction and autophagosome assembly. These molecular complexes include (1) ULK1-FIP200-Atg13 kinase complex, (2) Beclin1-VPS34 class III PI3-kinase complex, (3) Atg9-Atg2-Atg18 complex, and (4) the Atg5-Atg12-Atg16 and Atg8/LC3 conjugation systems. The important roles of these Atg complexes in regulating autophagy have been extensively reviewed [2].

## Autophagy in lipid metabolism

The liver plays a key role in lipid metabolism. It has been demonstrated that autophagy degradation can play a critical role in regulating intracellular lipid stores. Autophagosomes can transport the content of lipid droplets (LD) to the lysosome, in which lipids are degraded by the lysosomal acid lipase. This process is known as lipophagy [11]. Colocalization of LC3 with lipid droplet can be demonstrated in vivo and in vitro [11–14]. Lipid droplets and autophagic components can be associated during nutrient deprivation and inhibition of autophagy in cultured hepatocytes and mouse liver increases intracellular triglyceride level [11–13].

Other than autophagic molecules, recent studies found that dynamin 2 is involved in the regeneration of lysosomes, which is required for a sustained lipophagy [15]. Dynamin 2 is involved in membrane deformation and deletion of it causes LD accumulation and enlargement of autolysosomes. In addition, Rab7, located on LD, is required for lipophagy triggered by serum deprivation in hepatoma cell lines [16], and in 3T3-L1 cells where recruitment of lysosomal compartment to the LD is triggered by beta-adrenergic receptor activation [17]. In the latter case, the study indicated that lipophagy can also promote beta-adrenergic receptor-stimulated lipolysis [17]. Thus activation of beta-adrenergic receptor increases the association of LD with the autolysosomal membranes in 3T3-L1 cells. Furthermore inhibiting autophagy reduced lipolysis. Rab7 is also involved in the increased basal lipolysis induced by perilipin 1 knockdown.

In a different scenario, autophagy machinery has been implicated in the LD formation [18]. Lipid droplets formation accompanied by accumulation of triacylglycerol is largely suppressed in hepatocytes that cannot execute autophagy. LC3 was localized on the surface

of LDs and LC3-II (lipidation form) was fractionated to a perilipin- positive lipid fraction from livers under starvation. The authors argued that autophagy participates in LD formation. Taken together, the above studies indicate that autophagy may participate in lipid metabolism, particularly LD turnover, in multiple modes depending on the pathophysiological context.

## Autophagy in mitochondrial homeostasis

Mitochondria are important to cellular physiology. However, damaged mitochondria can be harmful by producing high levels of reactive oxygen species (ROS). Mitochondria homeostasis involves fissure and fusion, but the removal of damaged mitochondria depends on autophagy. Autophagic digestion of mitochondria was first noted in general non-selective autophagy [19]. Mitophagy, as a specific term for selective autophagic removal of mitochondria was first proposed by Lemasters and his colleagues [20, 21]. There has been a rapid progress in the study of mitophagy in the past few years, which has led to a great understanding of the molecular mechanisms, the pathophysiological role in development and in diseases, and the analytic approaches [22]. As mitochondria are essential organelles that regulate cellular energy metabolism and cell death, mitochondrial homeostasis has been linked to many pathophysiological conditions and diseases, including development, innate immunity aging, neurodegeneration, cancer, and tissue injury [22].

It seems that mitophagy in mammalian cells involves two distinguished steps: the induction of canonic Atg-dependent macroautophagy and mitochondrial priming [22, 23]. The induction of canonic autophagy requires Atg proteins, can involve mTOR suppression mediated by mitochondrial damage-generated ROS, and can also require AMPK activation triggered by ATP depletion. The priming of the mitochondria means that the damaged mitochondria are molecularly modified so that they become recognizable by the autophagosomes.

Molecules such as Parkin, an E3 ligase, Nix, a BH3-only Bcl-2 family protein, and FUNDC1, a phosphorylation target of Src kinase, have been implicated in mitophagy in different models [22]. Localization of Nix and Parkin is triggered by damaging signals whereas FUNDC1 is a mitochondrial membrane protein. While Nix and FUNDC1 can bind to LC3, thus serving as an adaptor between the autophagosome and the damaged mitochondria, Parkin is an E3 ligase, whose effect may be related to the ubiquitination and degradation of mitofusins. The latter can lead to mitochondrial fragmentation and thus facilitate mitophagy [24].

## Role of autophagy in alcoholic liver disease

Autophagy function may vary in alcoholic livers depending on the stage. Acute alcohol treatment activated hepatic autophagy in vivo and in cultured primary hepatocytes [12, 25]. Suppression of autophagy with pharmacologic agents or small interfering RNAs against Atg7 significantly increased hepatocyte apoptosis and liver injury [12, 25]. Chronic alcoholic treatment using the Lieber-DeCarli model also showed that suppression of

autophagy exacerbated liver injury while enhancement of autophagy improved the condition [13]. Autophagy therefore protects hepatocytes from the toxic effects of ethanol.

How does autophagy affect the pathogenesis of alcoholic fatty liver disease (ALD)? The pathological features ALD involve steatosis, inflammation, fibrosis and cirrhosis. It is generally considered that the pathogenesis of ALD is intimately related to oxidative stress, derived from reactive intermediates including acetaldehyde, increased NADH/NAD<sup>+</sup> ratio and ROS generation [26–29] (Figure 1). When ethanol is oxidized by alcohol dehydrogenase (ADH), acetaldehyde is generated and subsequently is oxidized by the mitochondrial aldehyde dehydrogenase. In both steps NADH was generated and is oxidized indirectly by mitochondrial electron transport system. The excessive amount of NADH and thus the reducing capacity in the mitochondrial electron transport system is thought to cause an increased leakage of mitochondrial reactive oxygen species (ROS), causing ethanol-induced oxidative stress. The oxidative stress is thought to be responsible for the functional and structural changes of mitochondria observed in ALD, including the altered oxidative phosphorylation, increased mitochondrial DNA strand breakage and deletion, altered mitochondrial proteome and altered mitochondrial dynamics [30–35].

The combination of increased oxygen free radicals and lipids triggered by ethanol metabolism will cause lipid peroxidation and the role of lipotoxicity and mitochondrial damage in ALD pathogenesis can be significant as the two can form a vicious cycle to further enhance oxidative damage. In this context, reducing fatty acids level and removing damaged mitochondria could thus mitigate liver injury (Figure 1). The esterified lipids are sequestered in lipid droplets and would be considered non-harmful, although de-esterification can occur, which would increase cellular free fatty acids level. Removal of lipid droplet may favor the equilibrium toward the esterification, reducing the intracellular level of toxic free fatty acids.

Indeed, it is found that autophagy induced by acute ethanol administration does not influence much the turnover of long-lived proteins under both basal and ethanol-stimulated condition [12]. But lipophagy and mitophagy are detected based on the colocalization of autophagosomes with the mitochondria or lipid droplets [12, 13]. Ethanol-induced mitophagy can be inhibited by anti-oxidants (Figure 1). Reactive oxygen species are generated during ethanol metabolism and/or due to mitochondria dysfunction. Inhibiting or promoting autophagy correspondingly worsens or ameliorates hepatic steatosis in acute and chronic ALD models [12, 13]. The protective effects of autophagy can slow down the progression of ALD, which often takes a long course to develop.

In general while we can understand the potential importance of mitophagy and lipophagy in ethanol-induced pathogenesis, we still do not understand how the selectivity is determined and how the selection process is accomplished. These events are generally applicable to selective autophagy toward all types of substrates, but are often not clearly differentiated. The understanding of these mechanisms can bring forward new therapeutic modalities to improve the disease outcome. While enhancing autophagy via general autophagy-inducing agents, such as rapamycin, has led to the reduction of cellular injury in ethanol intoxication and in other scenarios, this approach may have limitations. Specific enhancement of

selective autophagy relevant to particular settings may be the ultimate choice for a better control of individual disease processes.

## Dynamics of autophagy in ALD

### Activation of autophagy by ethanol

Ethanol can activate hepatic autophagy in vivo and in cultured primary hepatocytes [12, 25, Sid, 2013 #15] [36]. This activation requires ethanol metabolism and can thus require the activity of alcohol dehydrogenase and cytochrome P450 2E1 [12, Wu, 2012#10, 37, 38]. As discussed above, ethanol causes oxidative stress, which is the base for many ethanol-induced cellular changes. Oxidative stress seems to be responsible for autophagy induction. The main ethanol metabolite, acetaldehyde, a pro-oxidant and can trigger autophagy [37]. Anti-oxidants, such as N-acetyl cysteine (NAC) [12, 38] can suppress ethanol-induced autophagy. In addition, deletion of cyclophilin D, a major component of mitochondrial permeability transition pore, impaired ethanol-induced autophagy [39], possibly due to a reduction in permeability transition and thus reduced ROS generation.

Both suppression of mTORC1 [40] and activation of AMPK [36] can contribute to ethanol-induced autophagy under oxidative stress. Consistently, activation of autophagy by rapamycin reduces ethanol-induced liver injury [12, 13, 38]. Recently, it has been shown that FoxO3a is activated during ethanol treatment, and is responsible for the transcription of several autophagy genes [41]. Ethanol treatment of FoxO3a-deficient mice results in enhanced liver injury and steatosis. Resveratrol is well known to inhibit ethanol-induced liver injury and steatosis [42], and it can also activate autophagy via increased deacetylation of FoxO3a [41] and activation of SIRT1 [43]. Protection of ethanol-induced damage by globular adiponectin can also be mediated by its enhancement of autophagy via FoxO3a and AMPK [44].

It is likely that other mechanisms could contribute to autophagy activation as well. Ethanol treatment can lead to proteasome inhibition and ER stress, both of which are known to be linked to autophagy activation [45]. Suppression of proteasome causes compensatory activation of autophagy via ER stress-mediated UPR, in which the IRE-1 and JNK pathway are involved [46]. Indeed, proteasome activity is inversely correlated with autophagy activation in ethanol-treated cells [25]. Finally, metal elements, such as zinc, can also be critical for autophagy during ethanol exposure and under basal level in hepatoma cell lines [47]. Thus zinc addition in the medium stimulated autophagy. Ethanol treatment can change the expression of zinc transporters and metallothionein, thus activating autophagy.

### Autophagy suppression in ALD

Chronic alcoholic treatment using the Lieber-DeCarli model also showed an elevation of autophagy when ethanol was given at a lower level (accounting for 29% of the caloric need), but signs of suppression when ethanol was given at a higher level (accounting for 36% of the caloric need) [13]. However, in both cases, suppression of autophagy further exacerbated liver injury while enhancement of autophagy improved the condition.

Early studies indicated that the rate of hepatic protein degradation in ethanol-fed animals declined significantly [48], which might be due to declines in both proteasome and autophagy function, contributing to the development of hepatomegaly and the development of Mallory-Denk body (MDB). MDB is a characteristic of chronic alcoholic liver disease, and is biochemically positive for ubiquitin and p62/SQSTM1, which is found in protein aggregation and in autophagy deficiency. Indeed, by augmenting autophagy using rapamycin, an mTORC1 inhibitor, clearance of MDB can be achieved in a mouse model of MDB pathology [49].

Chronic alcoholic treatment could lead to decreases in both the number and the function of the lysosome, therefore reducing autophagic degradation [50]. Trafficking of exogenous proteins into the hepatocyte by endocytosis and the intracellular delivery of proteases to lysosomes can be both inhibited by ethanol consumption. Ethanol-induced suppression of autophagy may also result from alterations in hepatic amino acids levels. Because leucine is one of the strongest autophagy-inhibiting amino acids, higher levels of intrahepatic leucine may partially explain autophagic suppression in the ethanol-fed state.

Although direct evidence has yet to be sought, the functional status of autophagy may change during the development of alcoholic liver disease from the acute to the chronic status. Gradual loss of autophagy function during the disease process may occur and can in turn contribute to the exacerbation and the final presentation of the disease. This raises an interesting issue from the clinical point of view that an early intervention with autophagy-enhancing agents may help to block this vicious cycle and promote recovery following the cessation of alcohol drinking.

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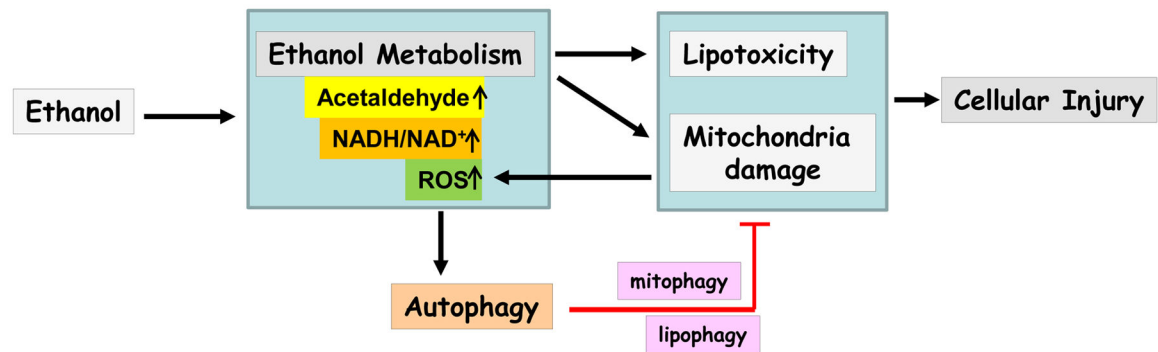
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**Figure 1. Mechanism of Autophagy in Protecting Liver from Ethanol-induced Toxicity**

Ethanol metabolism results in an increased amount of acetaldehyde, an increased NADH/NAD<sup>+</sup> ratio and an increased production of reactive oxygen species (ROS). The oxidative stress is further enhanced with the accumulation of lipids and mitochondrial damage, which can cause additional production of ROS and lipid peroxidation. Autophagy may protect the liver against ethanol-induced damage by removing lipids and damaged mitochondria.